Mitogen and stress-activated protein kinase (MSK1/2) regulates ischemia-induced neurogenesis and morphological maturation

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By

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Abstract

Pathophysiological conditions such as cerebral ischemia (stroke) trigger the production of new neurons in the hippocampus dentate gyrus. The functional significance of ischemia-induced neurogenesis is believed to be the regeneration of lost cells, thus contributing to post-stroke recovery. However, the cellular signaling mechanisms by which this process is regulated are still under investigation. Recent data from our lab indicate a role for mitogen and stress-activated protein kinases (MSK1/2) in the regulation of hippocampal progenitor cell proliferation after repeated seizure insult. In the present study we tested the hypothesis that MSK1/2 signaling is a regulator of ischemia-induced neurogenesis and neuronal morphological maturation. To test this hypothesis we induced transient ischemia by infusion of the potent vasoconstrictor endothelin-1 into the hippocampus. Following ischemic induction, hippocampal tissue was immunolabeled for bromodeoxyuridine (BrdU, a marker of newly dividing cells) and doublecortin (DCX, a marker for immature neurons). Detailed analysis of the granule cell layer (GCL) of the dentate gyrus indicates delayed maturation of newborn neurons in MSK1/2 null mice compared to wild type (WT) controls. Further, we performed a structural analysis of BrdU-tagged neurons born after ischemia in order to evaluate the maturation of the newly generated neurons, as a measurement of function capability. Taken together, these data suggest that MSK1/2 plays a significant role in the regulation of ischemia-induced neurogenesis and we will further investigate the morphological maturation of the newborn neurons.

Introduction

Stroke ranks No.4 as a leading cause of death in the U.S., and is a leading cause of long-term disability due to the cognitive, physical and emotional impairments it causes. 87% of all strokes are ischemic strokes (American Heart Association, 2012). Ischemia is a common occurring pathological condition associated with stroke, which damages the brain tissue due to insufficient oxygen supply as a result of reduced blood flow to the affected area. Post-ischemic recovery is difficult largely due to irreversible cell death in the ischemic core. Moreover, current available therapies aimed at the recovery of cells in the penumbra (potentially reversibly damaged cells in the surrounding area of the stroke site) are limited. Only approximately 10% of stroke survivors are able to recover almost fully (National Stroke Association, 2013). Most of this spontaneous recovery process happens within one week post ischemia (PI) and diminishes around 6 months PI, however permanent structural damages often persist (Taupin, 2006). The mechanisms of such striking recovery processes remain poorly understood.

Importantly, both global and focal ischemia promote neurogenesis in two regions of the adult brain: the subgranular zone (SGZ) of the hippocampus dentate gyrus (DG) and the subventricular zone (SVZ). Neuronal cells generated in the SGZ have been shown to play an important role in learning and memory (Deng et al., 2010). Moreover, cell tracking studies suggest that neurons born immediately after ischemia in the SGZ replace the lost neuronal cells by migrating to the granule cell layer in DG and differentiating into mature nerve cells (Taupin, 2006).

The mitogen activated protein kinase (MAPK) pathway plays an important role in the regulation of cellular activities by converting extracellular stimuli into various downstream cellular responses. MAPKs are found to be important in the regulation of gene expression,

proliferation, differentiation, metabolism, survival and apoptosis in all eukaryotic cells (Cargnello and Roux, 2011). Mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2) are effector kinases downstream of MAPK. MSK1/2 predominantly regulates nuclear substrates and regulates gene expression at different levels, which may affect both immunity and neuronal functions (Cargnello and Roux, 2011). MSK 1/2 is directly activated by MAPKs and by external stimulations including UV radiation, oxidative and chemical stress (Deak et al., 1998), and mediates the phosphorylation of cAMP response element binding protein (CREB), which affects neuronal cell differentiation and survival (Arthur, 2008). Previous work in our lab provides evidences that MSK 1/2 plays a significant role in seizure-induced neurogenesis, noted by a reduction in post-seizure neurogenesis, as well as impaired morphological maturation in the SGZ (Choi et al., 2012).

In the present study, we investigated the role of MSK 1/2 in the regulation of post-stroke neurogenesis and morphological maturation. We hypothesized that MSK 1/2 regulates the proliferation of newly generated neurons and also impacts the maturation of dendritic structures. Newborn cells in the hippocampal DG were examined in WT and MSK1/2 null mice following transient ischemia. Preliminary data indicate impaired morphological maturation of newborn neurons following ischemia in MSK1/2 null animals.

Materials and Methods

Animals

MSK1/2 null mice were kindly provided by Dr. J. Simon Arthur. WT and MSK1/2 null mice were crossed with a transgenic line expressing green fluorescent protein (GFP) under the control of the Thy1 promoter. DNA samples were obtained by tail digestion and phenol extraction. The genotype of mice was confirmed by using primer sets and PCR settings

(annealing temperature 66.4˚ C; 40 cycles of 30 seconds 94˚ C, 30 seconds 64˚ C, 60 seconds 72˚ C following 10 minutes 72˚ C before plate stays in 4˚ C until electrophoresis). Both male and female mice (6 weeks old) were used in the experiments. All procedures were approved by the IACUC and performed by authorized personnel in compliance with OSU animal welfare guidelines.

Endothelin-1 infusion

Mice were infused with endothelin-1 (ET-1) or physiological saline (2-3 mice per group). ET-1 is a potent vasoconstrictor that induces transient ischemia but is not directly neurotoxic (Bacigaluppi et al., 2010; Lustig et al., 1992). For the purposes of this study, this model was chosen for its ability to induce a relatively mild ischemic event in mice (Horie et al., 2008), thus allowing for a clear distinction of potentially subtle differences in ischemia-induced neurogenesis between WT and MSK1/2 null mice. ET-1 induced ischemia and cell death has been confirmed in our lab (Karelina et al., manuscript in preparation). Mice were anesthetized with an intraperitoneal (i.p.) injection of a cocktail containing ketamine (95.2 mg/kg) and xylazine (30.8 mg/kg) before being placed in a stereotaxic apparatus, the coordinates used were AP -2.06mm, ML 1.30mm, DV -2.00mm. All experimental group mice received a unilateral intrahippocampal infusion of 0.5µL ET-1 (1µg/uL in saline), the control group received saline in same amount. Tissue was collected four weeks PI for analysis of ischemia-induced neurogenesis and newborn neuron development; or six weeks PI for analysis of neuronal morphological maturation.

Bromodeoxyuridine injection

BrdU was injected i.p. at a dose of 50mg/kg. Mice were given a total of six injections on days six, seven and eight after ET-1 or saline infusion, two BrdU injections were administered six hours apart per day.

Histology and Immunolabling

Mice were transcardially perfused with 4% paraformaldehyde for fixation under anesthesia with ketamine/xylazine. Brains were collected and were post-fixed overnight in 4% PFA in 4˚ C before being placed in 30% sucrose solution with azide. Coronal sections (40μm) through the hippocampus were collected using a freezing microtome.

A three day protocol was used for double labeling of doublecortin (DCX) and BrdU, and triple labeling of Green Fluorescent Protein (GFP), BrdU and Deep Red Fluores-cing Anthraquinone Nr. 5(DRAQ-5). Selected sections were washed three times, 5 minutes each with 1X Phosphate-Buffered Saline/Triton-X (PBST) and blocked in 10% Normal Horse Serum (NHS) or Normal Goat Serum (NGS) for one hour. Sections were then incubated with anti-goat DCX (1:500) in 5% NHS, or anti-chicken GFP (1:2500) in 5% NGS in 4˚C overnight. On the consecutive day, washed tissues were placed into Alexa Fluor secondary antibody (donkey anti-goat 633 or goat anti-chicken 488) in 5% NHS/NGS for 2 hours. For BrdU labeling, tissue was denatured by incubation in 2N hydrochloric acid in 37˚ C for one hour, followed by a 10-minute neutralization with 0.05M boric acid (pH=8.5). After several washes, tissue was blocked in 10% NGS for one hour and then placed in anti-rat BrdU primary antibody (1:200) in 4˚C overnight. On the following day tissue was placed in goat anti-rat 594 secondary antibody (1:500) for 2 hours. DRAQ-5 (1:5000) in PBS-T was used as a counterstain before mounting and cover slipping.

Quantification

In the 4 week survival study, hippocampal slices were examined via confocal microscopy and z-stack pictures were taken in order to ensure cell processes to be captured completely. All BrdU positive cells in the dentate gyrus and double labeled BrdU-DCX cells were counted respectively. At least 100 cells per animal were counted. To determine the stages of neuron development in the DCX study, the four stages were defined using the criteria based on length of process and development of dendritic branching employed by Choi et al. (2012). Specifically, stage 1 is defined as cells with no noticeable process, stage 2 being cells have process but not extending into the molecular layer, stage 3 being cells have process extended into the molecular layer and stage 4 are cells have process and well developed branches extending into the molecular layer. Neurogenesis was determined as percent of double labeled BrdU/DCX cells relative to the total number of BrdU-positive cells. Data analysis was done by an individual blinded to the experimental conditions.

Results

MSK 1/2 null mice confirmation

Homozygous MSK1/2 null mice were generated by crossing $MSK1^{(+/-)}/2^{(+/-)}$ heterozygous mice. For the 6-week survival experiments, WT and MSK1/2 null mice were crossed with Thy1-GFP expressing mice. Genotype of mice was confirmed through PCR and gel electrophoresis stained with ethidium bromide (figure 1).

Development of immature newborn neurons

Animals sacrificed 4 weeks PI were perfused, and tissue containing hippocampus was stained for the presence of newborn immature neurons by the markers doublecortin (DCX) and BrdU. DCX is transiently expressed in developing neurons and expression of DCX decreases

sharply when neurons start to express mature markers (Brown et al., 2003), therefore DCX serves as a reliable marker for immature neurons. In order to determine the rate of neurogenesis, co-labeled DCX-BrdU were counted as a percentage of total BrdU-positive cells in the dentate gyrus. As shown in figure 2, the preliminary data suggest a sustained increase in neurogenesis 4 weeks post ischemia in both WT and MSK null mice compared to the saline control.

Given that ischemia resulted in a similar rate of neurogenesis in both WT and MSK nulls mice at this time point, these groups were further assessed to determine whether MSK regulates neuronal development. As such, co-labeled DCX- and BrdU-positive cells were manually identified and assigned to one of four different developmental stages using criteria adopted from a similar assessment in Choi et al (2012). As shown in figure 3, the preliminary data suggest a greater number of stage 1 cells (earliest stage of neuronal development) in MSK null mice, whereas in the WT animals, significantly more stage 4 cells (late stage of neuronal development) were found. These results are consistent with previously published data (Choi et al., 2012) on post-seizure development of neurons in MSK null and WT mice.

Morphological maturation of newborn neurons

In order to assess the morphological maturation of newborn neurons after ischemia, WT and MSK null mice were crossed with a transgenic line expressing green fluorescent protein (GFP) under the control of the Thy1 promoter. Thy1 is expressed in the cell body, axon, dendrites and dendritic spines of mature neurons throughout the CNS (Feng et al., 2000). At 6 week PI, we immunolabeled the tissue with GFP and BrdU; thus, co-labeled GFP and BrdU-positive cells indicate mature neurons that were born after ischemia. DraQ-5 was used as a counterstain. This technique will allow us to carry out an in-depth analysis of neuronal morphology, and importantly will allow us to do this specifically in those hippocampal cells that

were born immediately after ischemia. The initial focus of the analysis will be to study the dendritic spine density of granule cells in the dentate gyrus, and additional measures may include dendritic length and branching patterns. As shown in figure 4, we have thus far been able to successfully locate several GFP-BrdU co-labeled cells.

Discussion

In the present study, we investigated the role of MSK 1/2 in post-ischemia neurogenesis. We assessed neurons born immediately after ischemia and investigated neuronal development in WT and MSK 1/2 null mice in two different time points: four weeks and six week (PI), in order to study immature and mature neuronal development, respectively. Four weeks PI, our preliminary data suggest a similar rate of neurogenesis in WT and MSK null mice (figure 2). Further assessment of the stages of neuronal development indicates fewer late-stage immature neurons in MSK 1/2 null animals compared to the WT animals (figure 3). These data suggest that MSK 1/2 plays a role in regulating the development of immature neurons. In order to examine the morphological structure of mature neurons, in the six week PI study, we have established a method for successfully identifying mature neurons that were birth dated 6-8 days PI. Currently we are working on measurements of morphological maturation of these neurons by analyzing spine density, dendritic length and branching complexity.

Four weeks PI, surprisingly we found a similar rate of neurogenesis in WT and MSK null mice. These data appear to be in contrast with another related experiment in our lab in which BrdU data from animals sacrificed two days PI, showed a significant increase in hippocampal proliferation along the subgranular zone in WT mice compared to MSK1/2 null mice (Karelina et al., unpublished data). Ischemia-induced neurogenesis has been determined to peak at 7-11 days PI with 25% of newly produced cells dying shortly thereafter, and approximately 60% of the

remaining cells became neurons. (Sharp et al., 2002, Parent et al., 2003). Several studies provide evidence indicating that the majority of cells born immediately after ischemia fail to survive and generally die within two to five weeks PI (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003). This is most likely due to the unhealthy PI environment in which these newly generated cells are born into. Given that the difference in neurogenesis rate between WT and MSK1/2 null mice is present at an acute time point (2 days) after ischemia but not 4 weeks later may suggest a role for MSK 1/2 in the regulation of cell survival mechanisms.

In order to have a better understanding of the development of the surviving neurons born immediately after ischemia, we assigned the BrdU/DCX co-labeled cells into four different developmental stages as defined in Choi et al. (2012). DCX is involved in the regulation of cell migration (Dijkmans et al., 2010). The expression of DCX was found as early as in progenitor cells or neural stem cells, and it dramatically decreases to undetectable levels as neurons start to express mature makers (Brown et al., 2003). Our preliminary data suggest that after ischemia, MSK 1/2 null mice have a higher percentage of immature neurons in stage one (no noticeable processes) and a lower percentage in stage four (processes extending into the molecular layer with well-developed branching) compared to WT mice. These findings are consistent with previous observations in MSK1/2 null mice (Choi et al., 2012). Our data extend these earlier findings to indicate that MSK1/2 may play a role in the rate of neuronal maturation after ischemia. This is of particular importance as it is believed that ischemia-induced neurogenesis serves to replenish lost cells by forming new cells capable of fully integrating into the hippocampal network.

Although no obvious phenotype differences or health problems are observed in MSK 1/2 null mice (Wiggin et al., 2002), the deletion of MSK substantially reduces CREB

phosphorylation. MSK 1 is the major CREB kinase and mediates CREB phosphorylation (Deak et al., 1998; Arthur et al., 2004; Wiggin et al., 2002; Lee et al., 2003). In turn, CREB mediated transcription within the CNS is essential for cell survival, development and synaptic plasticity (Sakamoto et al., 2011). Recent data supports a role for MSK 1/2 as an intermediate of the MAPK/CREB regulated developmental signaling pathway within the SGZ (Choi et al., 2012). Thus, MSK likely affects ischemia-induced neurogenesis via a mechanism involving CREB phosphorylation.

In addition to understanding the role of MSK in early neuronal development, it is important to assess the morphology of neurons born immediately PI once they have matured. The ability of newborn neurons to achieve morphological maturation and establish connections to existing neighbor cells is critical for them to become a fully functional unit of the hippocampal circuit, and therefore contribute to learning and memory (Toni et al., 2008; Deng et al., 2010). In order to assess morphological maturation of neurons born PI, we crossbred WT and MSK 1/2 null mice with a Thy1 transgenic line which expresses GFP in dendritic spines, axons and cell body of neurons. To date we have successfully identified a small population of neurons co-labeled by BrdU and GFP. To investigate the morphology of newly generated neurons, we will be measuring the spine density, dendritic length and branching complexity of GCL neurons in WT and MSK1/2 null mice six weeks PI. These measurements will provide clues as to whether these newly produced neurons are able to integrate into the existing hippocampal circuit and thus achieve full function. Previous work in our lab indicates reduced dendritic branching as well as reduced spine density in MSK1 null mice compared to WT controls (Karelina et al., 2012). Our ongoing experiments are designed to extend these findings to determine a role for MSK1/2 in neuronal maturation after ischemia. Given the published results from our lab (Choi et al., 2012;

Karelina et al., 2012), we hypothesize lower spine density and branching complexity in MSK1/2 null mice after ischemia.

In conclusion, our preliminary data show a delay in the development of immature neurons that are born immediately after ischemia in MSK 1/2 null mice, suggesting a role for MSK 1/2 in neuronal development. We are also able to track newborn neurons after ischemia until they are fully mature, which will allow us to carry out extensive measurement of their morphological development and maturation. Taken together, these findings extend our understanding of the role of the MAPK signaling cascade in the regulation of proliferation and neurogenesis after ischemia. These preliminary data provide a foundation for further analysis of the MAPK effector kinase MSK and its role in the regulation of neuronal development after ischemia.

Figures

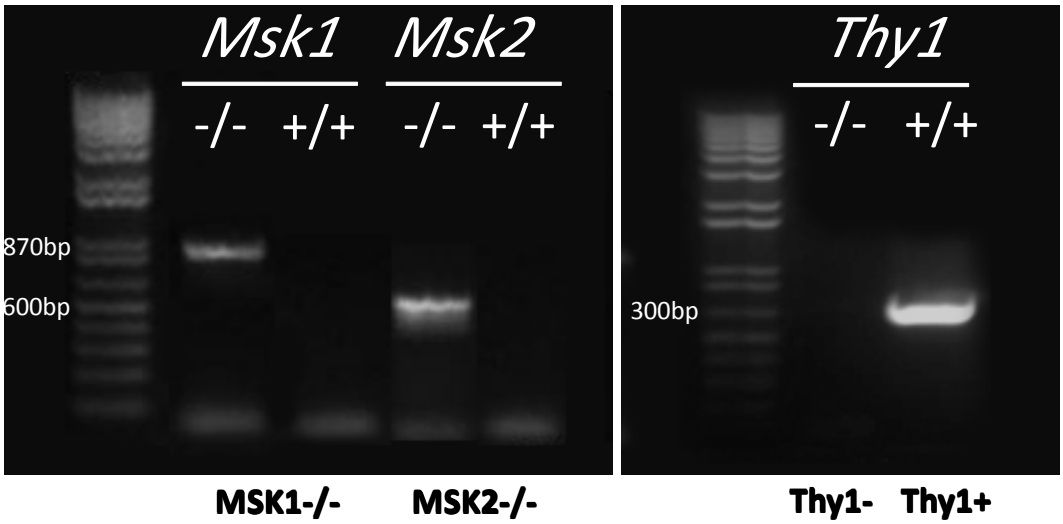

Figure 1. Genotyping results confirm the deletion of MSK 1/2. MSK1/2 and Thy1-GFP genetic modification was confirmed through PCR. DNA samples were obtained through tail digestion and extraction/purification. The DNA result from PCR was examined by using agarose gel electrophoresis staining with ethidium bromide.

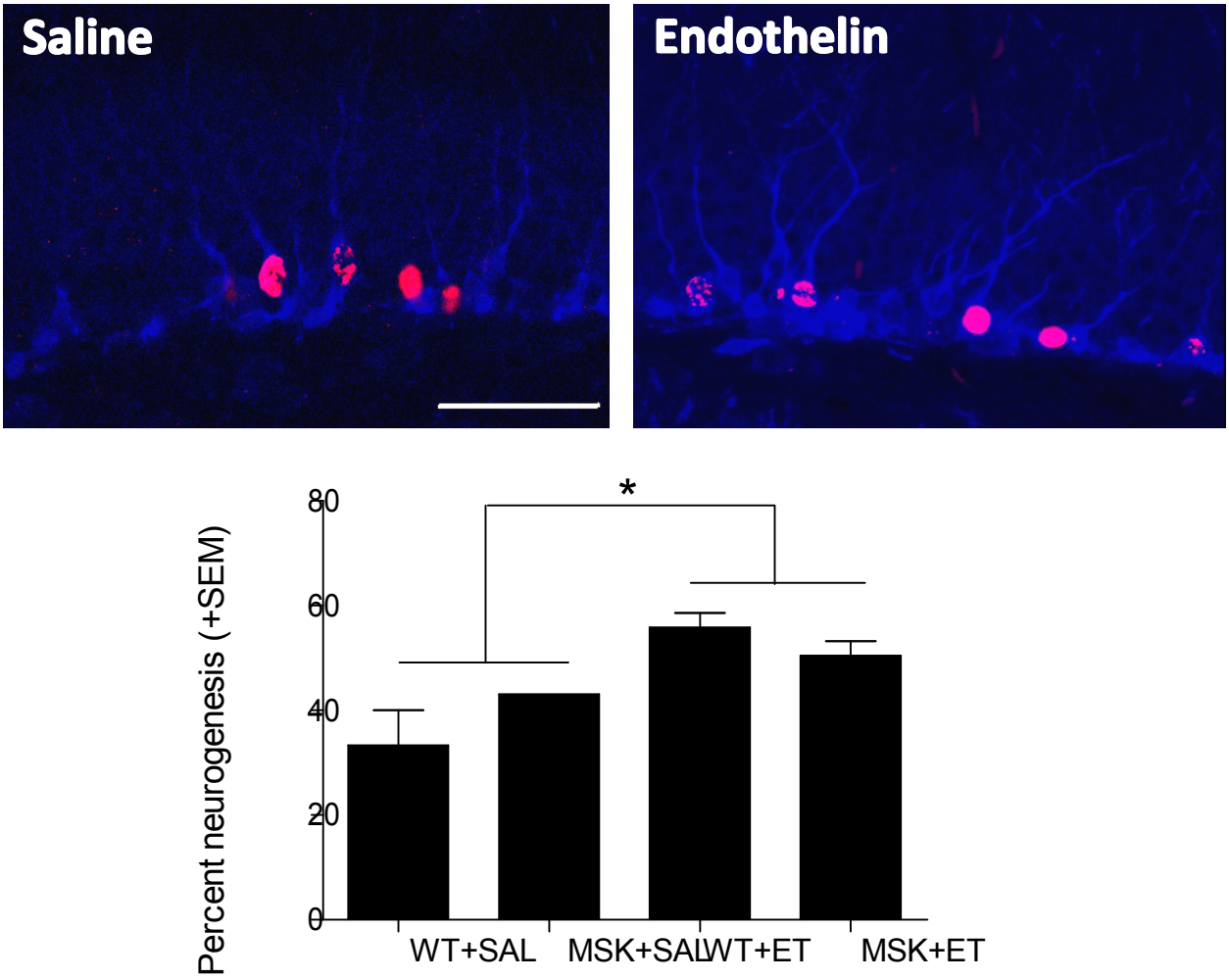

Figure 2. Ischemia-induced neurogenesis. Neurogenesis was assessed 4 weeks following ET-1 or saline infusion. Newborn neurons were labeled immunohistochemically using DCX (Blue: a marker of immature neurons) and BrdU (Red) antibodies. Data are presented as percent of BrdU-positive cells that are co-labeled with DCX. Preliminary data indicate a sustained increase in neurogenesis 4 weeks PI in both WT and MSK null mice. Scale bar = 50 μm.

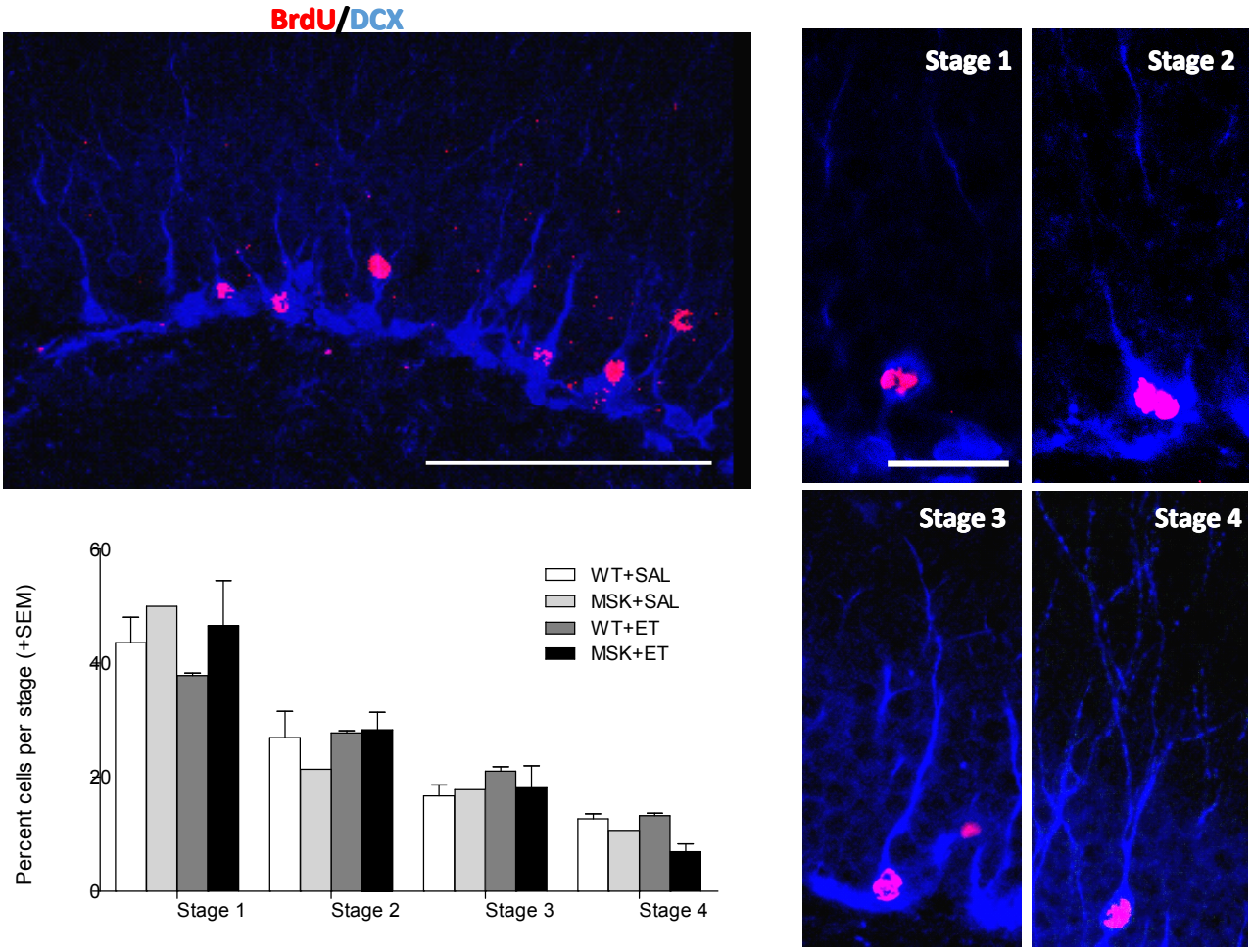

Figure 3. Stages of development of newborn neurons. Four weeks following ET-1 or saline infusion, DCXand BrdU-positive cells were assessed for stages of development. Stages were defined as previously reported (Choi et al, 2012). Briefly, stage 1 cells were defined as having no processes; stage 2 cells were defined as having short processes extending into the GCL; stage 3 cells were defined as those extending long processes into the molecular layer; and stage 4 cells were defined as having long processes and branches extending into the molecular layer. Preliminary data suggest an increase in the number of stage 1 cells, as well as a decrease in the number of stage 4 cells in MSK null mice compared to WT mice. Large image scale bar = 100μm, small images scale bar = 20 μm.

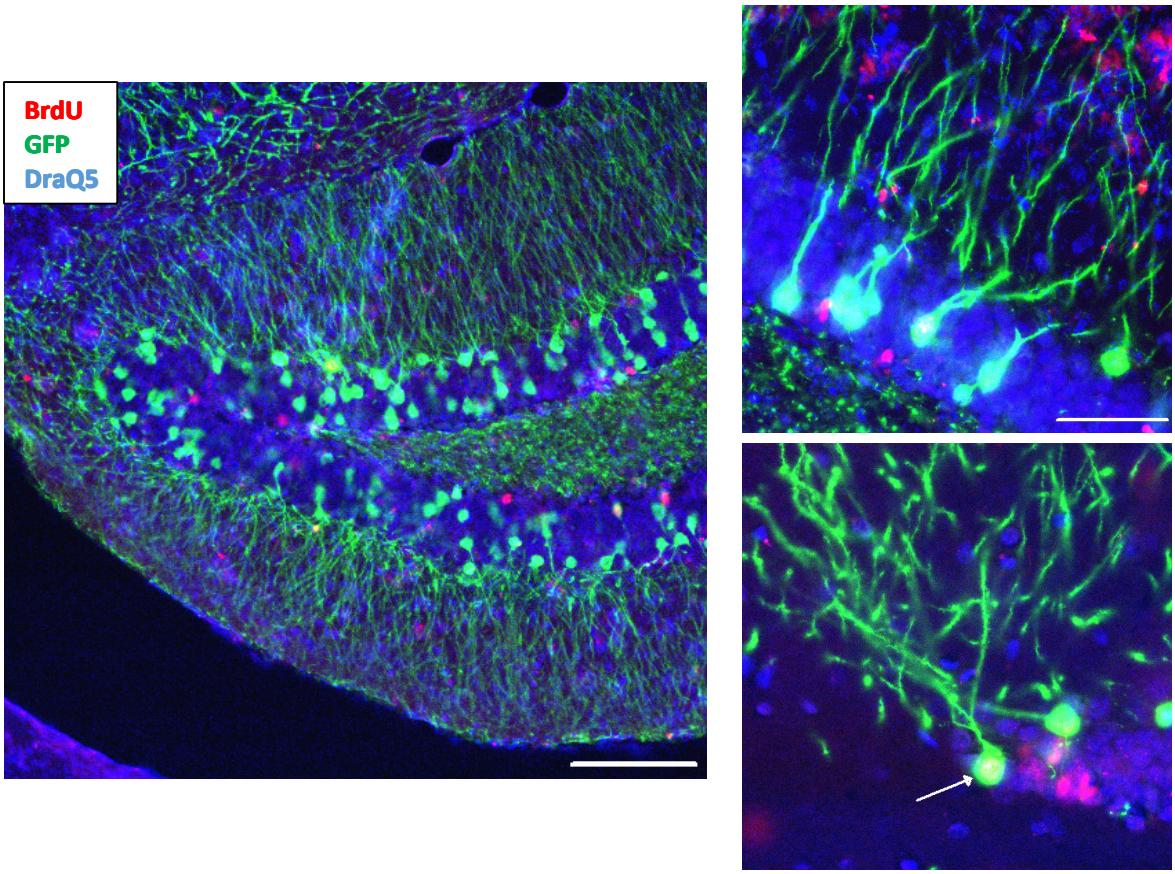

Figure 5. Morphological maturation of newborn neurons. Thy1-GFP expressing mice were sacrificed 6 weeks following ET-1 or saline infusion. Immunohistochemical labeling techniques were used to label GFP (green) and BrdU (red), DraQ5 (blue) was used as a nuclear counterstain. Shown are representative images of cells that co-label for GFP and BrdU. As a future direction, these images will be used to determine dendritic spine number as a measure of morphological maturation. Large image scale bar = 100μm, small images scale bar = 50 μm.

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